Nominated Activities for the Local Lymph Node Assay (LLNA): ICCVAM Preliminary Assessment and Recommendations

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1.0 INTRODUCTION

The Interagency Coordinating Committee for the Validation of Alternative Methods (ICCVAM) previously evaluated the validation status of the murine Local Lymph Node Assay (LLNA) as a stand-alone alternative method to the Guinea Pig Maximization Test (GPMT) and the Buehler Assay (NIH publication No. 99-4494; available at (*http://iccvam.niehs.nih.gov/methods/immunotox/llna.htm*). As a result of this evaluation, ICCVAM recommended the LLNA as a valid substitute for the guinea pig methods, for most testing situations. Subsequently, the LLNA was accepted within the United States by the Environmental Protection Agency, the Food and Drug Administration, and the Consumer Product Safety Commission (CPSC). In addition, an OECD Test Guideline (OECD TG 429) for the LLNA has been adopted by the 30 member OECD countries.

In January 2007, CPSC submitted a nomination to the National Toxicology Program Interagency Center for the Evaluation of Alternative Methods (NICEATM) (*http://iccvam.niehs.nih.gov/SuppDocs/submission.htm - nomination*) requesting that ICCVAM assess the validation status of:

- the LLNA as a stand-alone test for potency determinations (including severity) for the purpose of hazard classification;
- LLNA protocols that do not require the use of radioactive materials;
- the LLNA cut-down or "limit dose" procedure;
- the ability of the LLNA to test mixtures, aqueous solutions, and metals;
- the current chemical applicability domain of the LLNA.

On January 24, 2007, ICCVAM unanimously endorsed (1) developing performance standards for the LLNA, and (2) initiating a preliminary review of the available data and information associated with the CPSC nominated activities. A determination on which (if any) of the nominated activities will move forward will be made subsequent this review, and consideration of public and SACATM comments on the nominated activities. In anticipation of proceeding with an evaluation of these test methods, ICCVAM and NICEATM are proposing to convene a Panel that would review the usefulness and limitations of each of the LLNA protocols listed above. The Panel may also formulate conclusions on the adequacy of

any draft recommended performance standards, any proposed future validation studies, and draft standardized test method protocols.

2.0 NOMINATED ACTIVITY: ASSESSMENT OF THE VALIDATION STATUS OF THE LLNA AS A STAND-ALONE ASSAY FOR POTENCY DETERMINATIONS

2.1 Background

Based on the recommendations of ICCVAM and an independent scientific peer review panel (hereafter, Panel), the LLNA is now accepted as an alternative to the guinea pig maximization test and the Buehler test for assessing allergic contact dermatitis (ICCVAM 1999)[1]. However, the consensus of the Panel was that while the LLNA performed as well as the guinea pig tests for hazard identification of strong to moderate dermal sensitizing agents, it lacked strength in accurately predicting some weak sensitizers. The LLNA is therefore currently considered as a test method that provides quantitative data to support only a determination of the sensitization endpoint (i.e., yes/no decisions).

Although papers have been published showing correlations of dose-potency in animals with human potency, the validation status of such data have not been reviewed according to internationally recognized procedures. For this reason, CPSC recently requested that ICCVAM and NICEATM assess the current validation status of the LLNA as a stand-alone assay for potency determinations (including severity) for classification purposes.

2.2 Preliminary Review

NICEATM conducted a preliminary search[2] to determine the availability of published data relevant to the use of the LLNA to determine sensitization potency. Upon initial review of the search results, 38 published papers appeared to contain data relevant to the use of LLNA as a stand-alone assay for potency determinations. Based on the listed authors and their affiliations, these papers were published by seven different groups (31 by the groups of Basketter, Gerberick, and Kimber; two by the group of van Loveren; one by the group of

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[1]Available at: http://iccvam.niehs.nih.gov/methods/immunotox/immunotox.htm

[2]Search terms used and total number of citations identified for each method is provided in **Section 2.3**. References deemed to be most relevant for this analysis were reviewed further.

DeJong; and one each by the groups of Lalko, Greim, Schlede, and Schneider) and report results on approximately 619 substances. A detailed evaluation and assessment of performance will be prepared and included in a Background Review Document (BRD). Additional searches are ongoing and relevant data will be added to the database.

2.3 References Obtained During Preliminary Review

Search terms used:

PubMed: ("local lymph node" OR LLNA OR "Local Lymph Node" OR "Local lymph node") AND (potency OR potential) - 195 citations returned

Basketter DA and Kimber I. 2006. Predictive tests for irritants and allergens and their use in quantitative risk assessment. In: Contact Dermatitis, 4th ed., (Frosch PJ. , Menné T, Lepoittevin J-P, eds)., Berlin, Heigelberg: Springer, 179-187.

Basketter DA, McFadden J, Evans P, Andersen KE, Jowsey I. 2006. Identification and classification of skin sensitizers: Identifying false positives and false negatives. Contact Dermatitis 55(5):268-273.

Basketter DA, Andersen KE, Carola C, Van Loveren H, Boman A, Kimber I, et al. 2005. Evaluation of the skin sensitizing potency of chemicals by using the existing methods and considerations of relevance for elicitation. Contact Dermatitis 52(1):39-43.

Basketter DA, Clapp C, Jefferies D, Safford B, Ryan CA, Gerberick F, et al. 2005. Predictive identification of human skin sensitization thresholds. Contact Dermatitis 53(5):260-267.

Basketter DA, Cadby P. 2004. Reproducible prediction of contact allergenic potency using the local lymph node assay. Contact Dermatitis 50(1):15-17.

Basketter DA, Smith Pease CK, Patlewicz GY. 2003. Contact allergy: The local lymph node assay for the prediction of hazard and risk. Clinical and Experimental Dermatology 28(2):218-221.

Basketter DA, Wright ZM, Warbrick EV, Dearman RJ, Kimber I, Ryan CA, et al. 2001. Human potency predictions for aldehydes using the local lymph node assay. Contact Dermatitis 45(2):89-94.

106 Basketter DA, Blaikie L, Dearman RJ, Kimber I, Ryan CA, Gerberick GF, et al. 2000. Use of
107 the local lymph node assay for the estimation of relative contact allergenic potency. Contact
108 Dermatitis 42(6):344-348.

109 Basketter DA, Lea LJ, Cooper K, Stocks J, Dickens A, Pate I, et al. 1999. Threshold for
110 classification as a skin sensitizer in the local lymph node assay: A statistical evaluation. Food
111 and Chemical Toxicology 37(12):1167-1174.

112 Basketter DA, Rodford R, Kimber I, Smith I, Wahlberg JE. 1999. Skin sensitization risk
113 assessment: A comparative evaluation of 3 isothiazolinone biocides. Contact Dermatitis
114 40(3):150-154.

115 Basketter DA, Lea LJ, Dickens A, Briggs D, Pate I, Dearman RJ, et al. 1999. A comparison
116 of statistical approaches to the derivation of EC3 values from local lymph node assay dose
117 responses. Journal of Applied Toxicology 19(4):261-266.

118 Basketter DA, Roberts DW, Cronin M, Scholes EW. 1992. The value of the local lymph
119 node assay in quantitative structure-activity investigations. Contact Dermatitis 27(3):137-
120 142.

121 Betts CJ, Dearman RJ, Heylings JR, Kimber I, Basketter DA. 2006. Skin sensitization
122 potency of methyl methacrylate in the local lymph node assay: Comparisons with guinea-pig
123 data and human experience. Contact Dermatitis 55(3):140-147.

124 Betts CJ, Dearman RJ, Kimber I, Maibach HI. 2005. Potency and risk assessment of a skin-
125 sensitizing disperse dye using the local lymph node assay. Contact Dermatitis 52(5):268-272.

126 Dearman RJ, Wright ZM, Basketter DA, Ryan CA, Gerberick GF, Kimber I. 2001. The
127 suitability of hexyl cinnamic aldehyde as a calibrant for the murine local lymph node assay.
128 Contact Dermatitis 44(6):357-361.

129 DeJong WH, van Och FM, Den Hartog Jager CF, Spiekstra SW, Slob W, Vandebriel RJ, van
130 Loveren H. 2002. Ranking of allergenic potency of rubber chemicals in a modified local
131 lymph node assay. Toxicological Science 66(2):226-232.

132 Gerberick GF, Ryan CA, Dearman RJ, Kimber I. 2007. Local lymph node assay (LLNA) for
133 detection of sensitization capacity of chemicals. Methods 41(1):54-60.

Gerberick GF, Ryan CA, Kern PS, Schlatter H, Dearman RJ, Kimber I, et al. 2005. Compilation of historical local lymph node data for evaluation of skin sensitization alternative methods. Dermatitis 16(4):157-202.

Gerberick GF, Robinson MK, Ryan CA, Dearman RJ, Kimber I, Basketter DA, et al. 2001. Contact allergenic potency: Correlation of human and local lymph node assay data. American Journal of Contact Dermatitis 12(3):156-161.

Griem P, Goebel C, Scheffler H. 2003. Proposal for a risk assessment methodology for skin sensitization based on sensitization potency data. Regulatory Toxicology and Pharmacology 38(3):269-290.

Hilton J, Dearman RJ, Harvey P, Evans P, Basketter DA, Kimber I. 1998. Estimation of relative skin sensitizing potency using the local lymph node assay: A comparison of formaldehyde with glutaraldehyde. American Journal of Contact Dermatitis 9(1):29-33.

Jowsey IR, Basketter DA, Westmoreland C, Kimber I. 2006. A future approach to measuring relative skin sensitising potency: A proposal. Journal of Applied Toxicology 26(4):341-350.

Kimber I, Basketter DA. 1997. Contact sensitization: A new approach to risk assessment. Human and Ecological Risk Assessment (HERA) 3(3):385-395.

Kimber I, Basketter DA, Berthold K, Butler M, Garrigue JL, Lea L, et al. 2001. Skin sensitization testing in potency and risk assessment. Toxicological Sciences 59(2):198-208.

Kimber I, Basketter DA, Butler M, Gamer A, Garrigue JL, Gerberick GF, et al. 2003. Classification of contact allergens according to potency: Proposals. Food and Chemical Toxicology 41(12):1799-1809.

Kimber, I and Dearman, RJ. 1991. Investigation of lymph node cell proliferation as a possible immunological correlate of contact sensitizing potential. Food and Chemical Toxicology 29(2):125-129.

Kimber I, Gerberick GF, Basketter DA. 1999. Thresholds in contact sensitization: Theoretical and practical considerations. Food and Chemical Toxicology 37(5):553-560.

Kimber I, Hilton J, Dearman RJ, Gerberick GF, Ryan CA, Basketter DA, Scholes EW, Ladics GS. Loveless SE, House RV, Guy A. 1995. An international evaluation of the murine local lymph node assay and comparison of modified procedures. Toxicology 103:63-73

Lalko J, Api AM. 2006. Investigation of the dermal sensitization potential of various essential oils in the local lymph node assay. Food and Chemical Toxicology 44(5):739-746.

Lea LJ, Warbrick EV, Dearman RJ, Kimber I, Basketter DA. 1999. The impact of vehicle on assessment of relative skin sensitization potency of 1,4-dihydroquinone in the local lymph node assay. American Journal of Contact Dermatitis 10(4):213-218.

Loveless SE, Ladics GS, Gerberick GF, Ryan CA, Basketter DA, Scholes EW, et al. 1996. Further evaluation of the local lymph node assay in the final phase of an international collaborative trial. Toxicology 108(1-2):141-152.

Patlewicz G, Basketter DA, Smith CK, Hotchkiss SAM Roberts DW. 2001. Skin-sensitization structure-activity relationships for aldehydes. Contact Dermatitis 44: 331–336.

Ryan CA, Cruse LW, Skinner RA, Dearman RJ, Kimber I, Gerberick GF. 2002. Examination of a vehicle for use with water soluble materials in the murine local lymph node assay. Food and Chemical Toxicology 40(11):1719-1725.

Schlede E, Aberer W, Fuchs T, Gerner I, Lessmann H, Maurer T, et al. 2003. Chemical substances and contact allergy - 244 Substances ranked according to allergenic potency. Toxicology 193(3):219-259.

Schneider K, Akkan Z. 2004. Quantitative relationship between the local lymph node assay and human skin sensitization assays. Regulatory Toxicology and Pharmacology 39(3):245-255.

van Och FM, Vandebriel RJ, Prinsen MK, DeJong WH, Slob W, van Loveren H. 2001. Comparison of dose-responses of contact allergens using the guinea pig maximization test and the local lymph node assay. Toxicology 167(3):207-15. (Erratum in: Toxicology 2002. 170(3):228-230).

van Och FM, Slob W, DeJong WH, Vandebriel RJ, van Loveren H. 2000. A quantitative method for assessing the sensitizing potency of low molecular weight chemicals using a local

lymph node assay: employment of a regression method that includes determination of the undertainty margains. Toxicology 146(1):49-59.

Warbrick EV, Dearman RJ, Lea LJ, Basketter DA, Kimber I. 1999. Local lymph node assay responses to paraphenylenediamine: Intra- and inter-laboratory evaluations. Journal of Applied Toxicology 19(4):255-260.

2.4 Recommendation

A preliminary review indicates that there is sufficient information available to warrant a comprehensive review of the usefulness and limitations of using the LLNA to classify substances for sensitization potency. Therefore, a comprehensive BRD should be prepared that will evaluate the validation status the LLNA as a stand-alone assay for potency determinations (including severity) for classification purposes. This BRD will serve as the supporting information to be reviewed by an expert peer panel and ICCVAM.

ICCVAM has endorsed this activity as having high priority.

3.0 NOMINATED ACTIVITY: ASSESSMENT OF THE VALIDATION STATUS OF NON-RADIOACTIVE LLNA PROTOCOLS

3.1 Background

Based on the recommendations of ICCVAM and an independent scientific peer review panel, the LLNA is now accepted as an alternative to the guinea pig maximization test and the Buehler test for assessing allergic contact dermatitis (ICCVAM 1999)[3]. Since this review, there have been a number of modifications to the original protocol, as well as alternative LLNA testing strategies, that have been developed. In order for these modifications to be considered adequate for regulatory use, they must undergo a formal ICCVAM review of their usefulness and limitations relative to the traditional LLNA.

One of these modifications was developed to eliminate the need for using radioactivity, which is currently used in the traditional LLNA. A number of countries are either not permitted to use radioactivity or its use is very limited. In this regard a number of non-

[3]Available at: http://iccvam.niehs.nih.gov/methods/immunotox/immunotox.htm

radiolabeled endpoints are being explored to fulfill this need. Four types of methods predominate in the published literature: (1) methods that measure 5-bromo-2'-deoxyuridine (BrdU) incorporation, (2) methods employing flow cytometry to quantitate lymphocyte proliferation, (3) methods that measure cytokine release, and (4) a method that measures ear-draining lymph node weight and cell counts. The CPSC has requested that ICCVAM and NICEATM assess the current validation status of non-radioactive LLNA protocols.

3.2 Preliminary Review

NICEATM conducted a preliminary literature search[4] to determine the availability of relevant published data. A detailed evaluation and assessment of performance will be prepared and included in a BRD following a decision to carry out the nominated activities. The preliminary search identified reports exist for each of the four methods described above. Additionally, a number of posters relevant to these types of LLNA modifications were presented at the 2007 Society of Toxicology Annual Meeting in Charlotte, NC (March 25-29, 2007), which are included in the attached reference list.

Eleven papers, all of which were published after 1998 (i.e., after the original ICCVAM evaluation of the LLNA), reported the results of investigations of the BrdU incorporation method, involving the testing of 35 different substances. Based on the listed authors and their affiliations, the reviewed studies appear have been conducted in four different laboratories (one paper by Lee, one paper by Suda, six papers by Takeyoshi, and three papers by Yamano).

Four articles and three posters, all but one published after 1998, reported results for flow cytometric methods. The studies, conducted by four different groups (two papers by the groups of Gerberick and Kimber; one paper by the group of Lee, and one paper and three posters by the group of DeGeorge), involved testing of a total of 55 different substances.

Eight papers, by five groups, reported results for methods that measured cytokine release from lymph node cell suspensions. These studied tested 20 different substances. Of these papers, seven were published after 1998.

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[4]Search terms used and total number of citations identified for each method is provided in **Section 3.3**. References deemed to be most relevant for this analysis were reviewed further.

Four articles, all published after 1998, reported results for a method that measured draining lymph node weight and cell counts, involving the testing of 15 different substances. These studies were conducted in two different groups.

Additional searches are ongoing and relevant data will be added to the database.

3.3 References Obtained During Preliminary Review

Search terms used:

PubMed: (1) (non-RI OR nonRI OR nonrad*) AND (LLNA OR "Local Lymph Node" OR Local lymph node") - 16 citations returned

(2) (modified) AND (LLNA OR "Local Lymph Node" OR Local lymph node") - 37 citations returned

Scopus[5]*:* (TITLE-ABS-KEY(non-ri OR nonrad* OR nonri OR non-rad*) AND TITLE-ABS-KEY(llna OR "Local Lymph Node" OR "Local lymph node" OR "local lymph node")) - 18 citations returned

3.3.1 BrdU Incorporation

Lee JK, Park JH, Park SH, Kim HS, Oh HY. 2002. A nonradioisotopic endpoint for measurement of lymph node cell proliferation in a murine allergic contact dermatitis model, using bromodeoxyuridine immunohistochemistry. Jo urnal of Pharmacological and Toxicological Methods 48(1):53-61.

Suda A, Yamashita M, Tabei M, Taguchi K, Vohr HW, Tsutsui N, et al. 2002. Local lymph node assay with non-radioisotope alternative endpoints. Journal of Toxicological Sciences 27(3):205-218.

Takeyoshi M, Noda S, Yamasaki K, Kimber I. 2006. Advantage of using CBA/N strain mice in a non-radioisotopic modification of the local lymph node assay. Journal of Applied Toxicology 26(1):5-9.

[5]Abstract and citation database of research literature found at http://www.scopus.com/scopus/home.url.

Takeyoshi M, Iida K, Shiraishi K, Hoshuyama S. 2005. Novel approach for classifying chemicals according to skin sensitizing potency by non-radioisotopic modification of the local lymph node assay. Journal of Applied Toxicology 25(2):129-134.

Takeyoshi M, Noda S, Yamazaki S, Kakishima H, Yamasaki K, Kimber I. 2004a. Assessment of the skin sensitization potency of eugenol and its dimers using a non-radioisotopic modification of the local lymph node assay. Journal of Applied Toxicology 24(1):77-81.

Takeyoshi M, Noda S, Yamsaki K. 2004b. Differences in responsiveness of mouse strain against p-benzoquinone as assessed by non-radioisotopic murine local lymph node assay. Experimental Animals 53(2):171-173.

Takeyoshi M, Sawaki M, Yamasaki K, Kimber I. 2003. Assessment of statistic analysis in non-radioisotopic local lymph node assay (non-RI-LLNA) with α-hexylcinnamic aldehyde as an example. Toxicology 191(2-3):259-263.

Takeyoshi M, Yamasaki K, Yakabe Y, Takatsuki M, Kimber I. 2001. Development of non-radio isotopic endpoint of murine local lymph node assay based on 5-bromo-2'-deoxyuridine (BrdU) incorporation. Toxicology Letters 119(3):203-208.

Yamano T, Shimizu M, Noda T. 2005. Quantitative comparison of the results obtained by the multiple-dose guinea pig maximization test and the non-radioactive murine local lymph-node assay for various biocides. Toxicology 211(1-2):165-175.

Yamano T, Shimizu M, Noda T. 2004. Allergenicity evaluation of Bioban CS-1135 in experimental animals. Contact Dermatitis 50(6):339-343.

Yamano T, Shimizu M, Noda T. 2003. Allergenicity evaluation of p-chloro-m-cresol and p-chloro-m-xylenol by non-radioactive murine local lymph-node assay and multiple-dose guinea pig maximization test. Toxicology 190(3):259-266.

3.3.2 Flow Cytometry

Gerberick GF, Cruse LW, Ryan CA. 1999. Local lymph node assay: Differentiating allergic and irritant responses using flow cytometry. Methods: A Companion to Methods in Enzymology 19(1):48-55.

Humphreys NE, Dearman RJ, Kimber I. 2003. Assessment of cumulative allergen-activated lymph node cell proliferation using flow cytometry. Toxicological Sciences 73(1):80-89.

Kuhn U, Lempertz U, Knop J, Becker D. 1995. A new method for phenotyping proliferating cell nuclear antigen positive cells using flow cytometry: Implications for analysis of the immune response in vivo. Journal of Immunological Methods 179(2):215-222.

Lee JK, Park SH, Byun JA, Kim HS, Oh HY. 2004. Evaluation of lymphocyte subpopulations in draining lymph node cells following allergen and irritant. Environmental Toxicology and Pharmacology 17(2):95-102.

Reeder MK, Broomhead YM, DiDonato L, and DeGeorge GL. 2007. Use of an enhanced local lymph node assay to correctly classify irritants and false positive substances. The Toxicologist CD – An official journal of the Society of Toxicology. 96(S-1). Abstract #1136.

Reeder MK, Cerven DR, Gilotti AC, DeGeorge GL. 2006. Final validation of a flow cytometry-based local lymph node assay with enhanced immunophenotypic endpoints. [Poster] Presented at Society of Toxicology Annual Meeting, 5-9 March 2006, San Diego, Ca. USA.

Signs, SA and DeGeorge, GL. 2004. Application of a Modified LLNA to Petroleum-based Products: Dermal Sensitization Potential of Calcium Long-chain Alkyl Benzene Sulfonates. [Poster] Presented at Society of Toxicology Annual Meeting, 21 -25 March 2004, Baltimore, Md. USA.

3.3.3 Cytokine Release

Azam P, Peiffer JL, Ourlin JC, Bonnet PA, Tissier MH, Vian L, et al. 2005. Qualitative and quantitative evaluation of a local lymph node assay based on ex vivo interleukin-2 production. Toxicology 206(2):285-298.

De Jong WH, Tentij M, Spiekstra SW, Vandebriel RJ, Van Loveren H. 2002. Determination of the sensitising activity of the rubber contact sensitisers TMTD, ZDMC, MBT and DEA in a modified local lymph node assay and the effect of sodium dodecyl sulfate pretreatment on local lymph node responses. Toxicology. 176:123-134.

Hariya T, Hatao M, Ichikawa H. 1999. Development of a non-radioactive endpoint in a modified local lymph node assay. Food and Chemical Toxicology 37(1):87-93.

Hatao M, Hariya T, Katsumura Y, Kato S. 1995. A modification of the local lymph node assay for contact allergenicity screening: Measurement of interleukin-2 as an alternative to radioisotope-dependent proliferation assay. Toxicology 98(1-3):15-22.

Jong KL, Jae HP, Eom JH, Hyung SK, Hye YO. 2005. Modulation of intracellular cytokines in draining lymph node cells following allergen and irritant. Environmental Toxicology and Pharmacology 20(1):225-232.

van den Berg FA, Baken KA, Vermeulen JP, Gremmer ER, van Steeg H, van Loveren H. 2005. Use of the local lymph node assay in assessment of immune function. Toxicology. 211(1-2):107-114.

Vandebriel RJ, De Jong WH, Hendriks JJA, Van Loveren H. 2003. Impact of exposure duration by low molecular weight compounds on interferon-γ and interleukin-4 mRNA expression and production in the draining lymph nodes of mice. Toxicology 188(1):1-13.

Vandebriel RJ, De Jong, Spiekstra SW, Van Dijk M, Fluitman A, garassen J, Van Loveren. 2000. Assessment of preferential T-helper 1 or T-helper 2 induction by low molecular weight compounds using the local lymph node assay in conjunction with RT-PCR and ELISA for interferon-gamma and interleukin-4. Toxicology and Applied Pharmacology 162(2)77-85.

3.3.4 Ear-draining Lymph Node Weight/Cell Counts.

De Jong WH, Tentij M, Spiekstra SW, Vandebriel RJ, Van Loveren H. 2002. Determination of the sensitising activity of the rubber contact sensitisers TMTD, ZDMC, MBT and DEA in a modified local lymph node assay and the effect of sodium dodecyl sulfate pretreatment on local lymph node responses. Toxicology 176:123-134.

Ehling G, Hecht M, Heusener A, Huesler J, Gamer AO, Van Loveren H, et al. 2005a. An European inter-laboratory validation of alternative endpoints of the murine local lymph node assay: First round. Toxicology 212(1):60-68.

Ehling G, Hecht M, Heusener A, Huesler J, Gamer AO, Van Loveren H, et al. 2005b. An European inter-laboratory validation of alternative endpoints of the murine local lymph node assay: 2nd round. Toxicology 212(1):69-79.

Hans-Werner V, Jurgen AH. 2005. The local lymph node assay being too sensitive? Archives of Toxicology 79(12):721-728.

3.3.5 ATP Measurement

Yoshimura I, Idehara K, Omori T, Kojima H, Sozu T, Arima K, et al. 2007. Validation of LLNA-DA assay for assessing skin sensitization potential. The Toxicologist CD – An official journal of the Society of Toxicology. 96(S-1). Abstract #1135.

3.3.6 CD86/CD54 Measurement

Ashikaga T, Kosaka N, Sono S, Hitoshi S, Hiroyuki, Itagaki H. 2007. Comparative evaluation of the in vitro skin sensitization test; Human cell line activation test (h-CLAT) with LLNA and human data. The Toxicologist CD – An official journal of the Society of Toxicology. 96(S-1). Abstract #1144.

3.3.7 B Cell Measurement

Lalko J, Api A. 2007. Use of a B cell marker to discriminate between the irritant and allergenic potential of d-Lionene. The Toxicologist CD – An official journal of the Society of Toxicology. 96(S-1). Abstract #1145.

3.4 Recommendation

A preliminary review indicates that there is sufficient information available to warrant a comprehensive review of the usefulness and limitations of non-radiolabeled modifications to the LLNA. Therefore, a comprehensive BRD should be prepared to support evaluation of the validation status of non-radiolabeled LLNA methods. This BRD will serve as the supporting information to be reviewed by an expert peer panel and ICCVAM.

ICCVAM has endorsed this activity as having high priority.

4.0 NOMINATED ACTIVITY: ASSESSMENT OF THE VALIDATION STATUS OF THE USE OF THE LLNA TO TEST MIXTURES, AQUEOUS SOLUTIONS AND METALS

4.1 Background

Based on the recommendations of ICCVAM and an independent scientific peer review panel (hereafter, Panel), the LLNA is now accepted as an alternative to the guinea pig maximization test and the Buehler test for assessing allergic contact dermatitis (ICCVAM 1999)[6]. As described in the ICCVAM report, a limitation of the LLNA was its inability to identify metal salts as contact allergens. However, the Panel recognized that studies in the literature suggest that the use of alternative vehicles can improve sensitivity of the LLNA for metal salts.

Additionally, the LLNA was evaluated for testing individual chemical substances. The usefulness and limitations of the LLNA for testing mixtures, especially aqueous mixtures, has not been adequately evaluated. However, data available in the literature demonstrate the wide variability in dose-potency (up to 20-fold) of chemical substances when applied with different solvents when tested using the LLNA. Adjuvant chemicals can potentiate or diminish the strength of sensitizing ingredients.

Based on these apparent data gaps, CPSC has recently requested that ICCVAM and NICEATM assess the current validation status of the use of the LLNA to test mixtures, aqueous solutions and metals.

4.2 Preliminary Review

NICEATM conducted a preliminary search[7] to determine the availability of published data relevant to these types of substances. A detailed evaluation and assessment of performance will be prepared and included in a BRD.

The search for LLNA studies with mixtures indicated that five papers reported results. The studies described in the papers were produced by four different groups (one paper each from

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[6]Available at: http://iccvam.niehs.nih.gov/methods/immunotox/immunotox.htm

[7]Search terms used and total number of citations identified for each method is provided in **Section 4.3**. References deemed to be most relevant for this analysis were reviewed further.

the groups of Lalko, Nakamura, Selgrade; two papers from the group including Skold). A preliminary review indicated that 15 different mixtures were evaluated among these five studies. When the review was limited to the four articles published after 1998 (i.e., after the original ICCVAM evaluation of the LLNA) the number of different mixtures tested was 12.

A search for studies on metal-containing substances yielded 18 published papers produced by nine different groups (four by the group including Basketter, Gerberick, and Kimber; five by the group including Ikarashi; two by the group of Hostynek and Maibach; two by the group of Nemery; and one each by the groups of Andersen, Stokes, Noda, Ichikawa, and Tinkle). A preliminary review of these papers indicated that there were 20 different substances evaluated. When the review was limited to those 10 papers published after 1998, the total number of substances evaluated was reduced to 19 different metal-containing substances.

One paper, published in 2002, was reviewed which reported results obtained with an alternate vehicle used to test water-soluble materials. Additionally, one poster was presented at the 2007 Society of Toxicology Annual Meeting in Charlotte, NC (March 25-259, 2007) that discussed the results of studies using an alternate vehicle (Pluronic® L92 block copolymer surfactant) in the LLNA.

Additional searches in public databases (e.g., search for alternative vehicles) are ongoing and any relevant data will be added to the database.

4.3 References Obtained During Preliminary Review

4.3.1 Mixtures

Search terms used:

Scopus[8]: (TITLE-ABS-KEY(mixture* OR formulat*) AND TITLE-ABS-KEY(llna OR "local lymph node")) - 24 citations returned

Lalko J, Api AM. 2006. Investigation of the dermal sensitization potential of various essential oils in the local lymph node assay. Food and Chemical Toxicology 44(5):739-746.

[8]Abstract and citation database of research literature found at http://www.scopus.com/scopus/home.url.

Nakamura A, Kanazawa Y, Sato H, Tsuchiya T, Ikarashi Y, De Jong WH, et al. 2003. Evaluation of allergic potential of rubber products: Comparison of sample preparation methods for the testing of polymeric medical devices. Journal of Toxicology - Cutaneous and Ocular Toxicology 22(3):169-185.

Sailstad DM, Tepper JS, Doerfler DL, Qasim M, Selgrade MK. 1994. Evaluation of an azo and two anthraquinone dyes for allergic potential. Fundamental and Applied Toxicology 23(4):569-577.

Skold M, Karlberg AT, Matura M, Borje A. 2006. The fragrance chemical beta-caryophyllene - Air oxidation and skin sensitization. Food and Chemical Toxicology 44(4):538-545.

Skold M, Borje A, Harambasic E, Karlberg AT. 2004. Contact allergens formed on air exposure of linalool. Identification and quantification of primary and secondary oxidation products and the effect on skin sensitization. Chemical Research in Toxicology 17(12):1697-1705.

4.3.2 Metals

Search terms used:

Scopus[9]: (TITLE-ABS-KEY(llna OR "local lymph node") AND TITLE-ABS-KEY(metal* OR aqueous)) - 37 citations returned

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Yamano T, Shimizu M, Noda T. 2006. Allergenicity and cross-reactivity of naphthenic acid and its metallic salts in experimental animals. Contact Dermatitis 54(1):25-28.

4.3.3 Aqueous Solutions

Search terms used:

Scopus[10]: (TITLE-ABS-KEY(llna OR "local lymph node") AND TITLE-ABS-KEY(metal* OR aqueous)) - 37 citations returned

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4.4 Recommendation

A preliminary review indicates that there is sufficient information available to warrant a comprehensive review of the usefulness and limitations of the LLNA for testing metals, mixtures, and aqueous solutions. Therefore, a comprehensive BRD should be prepared that will evaluate the validation status the LLNA as a stand-alone assay to test these types of

[10]Abstract and citation database of research literature found at http://www.scopus.com/scopus/home.url.

materials. This BRD will serve as the supporting information to be reviewed by an expert peer panel and ICCVAM. This review could result in expanding the applicability domain of the LLNA and therefore further reduce and refine animal use for skin sensitization testing.

ICCVAM has endorsed this activity as having high priority.

5.0 NOMINATED ACTIVITY: ASSESSMENT OF THE VALIDATION STATUS OF THE LLNA LIMIT TEST

5.1 Background

Based on the recommendations of ICCVAM and an independent scientific peer review panel, the LLNA is now accepted as an alternative to the guinea pig maximization test and the Buehler test for assessing allergic contact dermatitis (ICCVAM 1999)[11]. Since this review, there have been a number of modifications to the original protocol, as well as alternative LLNA testing strategies, that have been developed. In order to determine if these modifications are appropriatefor regulatory use, their usefulness and limitations relative to the traditional LLNA must be evaluated.

One of these protocol modifications reduces the number of dose groups to two (a single high-dose group and a concurrent vehicle control group) instead of the three to five dose groups used in the traditional LLNA. This modified version of the LLNA is referred to as the "LLNA limit test" or the "cut-down screen" in the published literature. CPSC has recently requested that ICCVAM and NICEATM assess the current validation status of the LLNA limit test.

5.2 Preliminary Review

NICEATM conducted a preliminary literature search to determine the availability of published data relevant to the LLNA limit test. A detailed evaluation and assessment of comparative performance will be prepared and included in a BRD. The preliminary search indicated one report exists. The report, published in 2006, employed a retrospective analysis of an existing LLNA database of 211 different chemicals.

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[11]Available at: http://iccvam.niehs.nih.gov/methods/immunotox/immunotox.htm

More recently, two posters presented at the 2007 Society of Toxicology Annual Meeting in Charlotte, NC (March 25-259, 2007) discussed the LLNA limit test. Also at the SOT meeting, a presentation titled "Integrated Systems and a Modified Local Lymph Node Assay" discussed the use of the LLNA limit test to identify skin sensitizers was given by Dr. David Basketter.

5.3 References Obtained During Preliminary Review

Basketter D, Patlewicz G, Gerberick F, Ryan C, Kern P, Betts C, et al. 2007. Identification of skin sensitizing chemicals in a reduced LLNA. The Toxicologist CD – An official journal of the Society of Toxicology. 96(S-1). Abstract #1139.

Basketter D. 2007 Integrated systems and a modified local lymph node assay. The Toxicologist CD – An official journal of the Society of Toxicology. 96(S-1).Abstract #592.

Chaney J, Rayn C, Kern P, Patlewicz G, Basketter D, Betts C, et al. 2007. The impact of reducing animal numbers in the local lymph node assay. The Toxicologist CD – An official journal of the Society of Toxicology. 96(S-1). Abstract #1140.

Kimber I, Dearman RJ, Betts CJ, Gerberick GF, Ryan CA, Kern PS, et al. 2006. The local lymph node assay and skin sensitization: A cut-down screen to reduce animal requirements? Contact Dermatitis 54(4):181-185.

5.4 Recommendation

While a preliminary review suggests that limited information is currently available, the potential impact on animal savings that could be achieved by using the LLNA limit test approach warrants that a comprehensive review of the usefulness and limitations of this approach be conducted. Therefore, a comprehensive BRD should be prepared that will evaluate the validation status of this approach, and this BRD will serve as the supporting information to be reviewed by an expert peer panel and ICCVAM.

ICCVAM has endorsed this activity as having high priority.

6.0 SUMMARY

Based on the preliminary reviews described above, it appears that there is sufficient information and rationale to support moving forward with a comprehensive review of these modifications to the LLNA protocol and the manner in which LLNA data are used for hazard classification. Therefore, a single BRD will be compiled that encompasses all four of the nominated activities outlined above, and this BRD will then serve as the basis for review and recommendations by an independent expert peer review panel (the Panel). The conclusions and recommendations of the Panel will be forwarded to ICCVAM for consideration in developing ICCVAM test method recommendations.